on both double- and single-stranded (ds and ss) DNA in presence of ATP, or a non-hydrolysable analog such as ATPγS. We here present studies of RecA-filaments, formed on either ds- or ssDNA, in a nanofunnel. The funnel geometry used allows probing the extension of a single filament at different channel dimensions, which in turn enables determination of the persistence length. The value we obtain for RecA on dsDNA, 1.15 ± 0.30 μm, agrees well with the literature. It is not straight-forward to obtain ssDNA that is long enough to allow studies in nanochannels. However, using rolling circle amplification (RCA) we were able to create ssDNA that is tens of thousands of bases. Using this ssDNA we estimate the persistence length to 1.44 ± 0.68 μm, slightly larger than what has been obtained for RecA-ssDNA filaments with conventional techniques.
Importantly, our experiments are performed in solution without the need to attach the DNA or protein to any "handles". This in turn means that the approach is directly applicable to most DNA-protein complexes and potentially also DNA-protein complexes extracted from cells.

2169-Plat
Dynamics of NAP1-Assisted Nucleosome Assembly Imaged with High-Speed Atomic Force Microscopy
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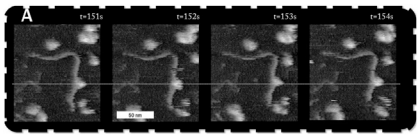
Nucleosome assembly is a vital part of chromatin maintenance for all eukaryotic cells. The histone chaperone NAP1 is one of the proteins involved in this process, guiding histones into the nucleus and assembling them into nucleosomes without use of ATP or other energy sources. It is known that NAP1 first brings H3H4 histone tetramers to the DNA to form a tetrasome, and in a second step H2a and H2B histones are added, but mechanistic insight into this process is still lacking. We use high-speed AFM to image NAP-1 assembled tetrasomes in vitro with spatial and temporal resolutions of the order of 1 nm and 1 s. We observe a rich palette of dynamical processes, among which are spontaneous tetrasome dissociation, cluster formation and a transient association between bare DNA and NAP1. One intriguing observation is the hopping of a tetrasome between two stable positions spaced only a few nanometers apart. We believe this motion can be ascribed to a re-orientation of the DNA around the histones, a phenomenon that we also observed in magnetic tweezer single molecule assays.



2170-Plat
Characterizing the Structure and Function of the N-Terminus of Schizosaccharomyces Pombe Cdc5, a Pre-Mrna Splicing Factor
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The spliceosome is a dynamic macromolecular machine composed of 5 small nuclear ribonucleoproteins (snRNPs), the Nineteen Complex (NTC), and other proteins that catalyze the removal of introns from pre-mRNA. The NTC is a highly conserved sub-complex of the spliceosome consisting of approximately fifteen proteins and is required for the transition of the spliceosome from an inactive to activated complex. However the mechanism of NTC function in this process is not understood. Schizosaccharomyces pombe Cdc5, a core subunit of the NTC, is an essential protein that contains two highly conserved N-terminal Myb repeats, a N-terminal non-canonical Myb-like repeat (MLR) as well as a less well conserved C-terminus. Both in vivo and in vitro studies have shown that the C-terminus of Cdc5 directly interacts with other NTC components; however, a specific function for the N-terminus of Cdc5 in pre-mRNA splicing has not been determined.
Our goal is to understand the specific role(s) of Cdc5 in NTC function and pre-mRNA splicing. Using a combination of yeast genetics, NMR secondary structure analysis, Chemical Shift (CS) Rosetta modeling, and an RNA binding assay we have shown the MLR is structurally distinct from the canonical Myb fold, that the N-terminus of Cdc5 preferentially binds double stranded RNA, and defined the importance the of the N-terminal Cdc5 Myb repeats and MLR in fission yeast.

2171-Plat
Condensation of DNA Mediated by the Bacterial Centromere Binding Protein Spo0J/ParB
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The condensation and dynamic re-organization of the chromosome is crucial to the cell cycle of all living organisms. In B. subtilis, the centromere binding protein Spo0J/ParB has recently been implicated in the recruitment of condensins at parS sequences close to the origin of replication [1, 2]. We have studied the binding of Spo0J to DNA molecules using magnetic tweezers. At reduced forces and high Spo0J concentrations, we observe a progressive condensation of the tethered DNA molecule. The condensation process is reversible upon increasing the force or removal of the protein, and shows a maximum stalling force of 3 pN at 8 μM Spo0J. Similar condensation curves were obtained for DNA substrates that either did or did not contain parS sequences, suggesting a non-specific binding mode of SpoOJ in agreement with complementary bulk studies. Control experiments using short competitor oligonucleotides confirmed that the condensation was Spo0J-mediated. Experiments using the freely orbiting tweezers technique and torsionally constrained DNA molecules show that the condensation process is accompanied by a preferred sense of rotation of the holding bead, but both clockwise and counter-clockwise rotations were found. We propose a model in which condensation is induced by interactions between neighbouring Spo0J proteins and these interactions are affected by the Brownian motion of the DNA tether.
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2172-Plat
Architectural Role of HMO1 in Bending, Bridging and Compacting DNA
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HMO1 proteins are abundant Saccharomyces cerevisiae (yeast) High Mobility Group Box (HMGB) proteins. HMGB proteins are nuclear proteins that are known to have an architectural function. HMO1 possesses two HMGB box domains. It has been reported that double box HMGB proteins induce strong bends upon binding to DNA. It is also believed that they play an essential role in reorganizing chromatin and therefore are likely to be involved in gene activation. To characterize DNA binding we combine single molecule stretching experiments and AFM imaging of HMO1 proteins bound to DNA. By stretching DNA bound to HMO1, we determine the dissociation constant and the cooperative parameter. Furthermore, we learn that HMO1 proteins form loops, and by pulling on these loops, we characterize the size of these loops and the average force to break a loop. Stretching experiments enable us to study the dynamics of loop formation and the time for reformation of these loops after they are pulled apart. AFM images are used to probe the conformations of individual HMO1-DNA complexes. The results show that at lower concentrations, HMO1 preferentially binds to the ends of the double helix and links separate DNA strands, forming bridges. At higher concentrations HMO1 induces formation of a complex network that reorganizes DNA. Taken together, these results suggest that both HMG boxes of HMO1 participate in multiple functions in vivo.

2173-Plat
Direct Visualization of DNA Dynamics During the Telomerase Catalytic Cycle Reveals the Function of a Conserved Telomerase Domain
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Telomerase is an enzyme that maintains the ends of eukaryotic chromosomes and is important to our understanding of both aging and cancer. The telomerase catalytic core contains both a conserved protein subunit known as telomerase reverse transcriptase (TERT), and a conserved RNA known as telomerase RNA (TER). Telomerase recognizes its DNA substrate by

base-complementarity with a region of TER, and extends the DNA using TERT's reverse transcriptase activity. Structural, functional, and kinetic studies of telomerase have been limited by its poor expression in vivo and in heterologous systems, as well as its structural heterogeneity. For this reason, this is an ideal system to study at the single molecule level, as single molecule assays do not require large amounts of material and can parse out different sub-populations of molecules. Our lab has developed a single-molecule Forster resonance energy transfer (smFRET) assay in order to monitor structural changes in the telomerase enzyme during its activity. We have used this assay in combination with several telomerase mutants to conduct structural and mechanistic studies of telomerase function. Our results demonstrate that a conserved telomerase N-terminal domain (TEN domain) stabilizes duplex formation between telomerase RNA and its DNA substrate. This TEN domain functions by altering the equilibrium between the duplex (active) state and a previously unknown alternative (inactive) binding conformation. Furthermore, we demonstrate that mutants that stabilize the alternative conformation have severe defects in telomerase activity. The discovery of this inactive alternative state suggests that stabilizing this conformation could be a useful of target of telomerase inhibitors for future cancer treatment.

2174-Plat

Targeting and Degradation of Viral DNA by the CRISPR-Cas System of Escherichia Coli

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Bacteria and archaea maintain a history of viral infections by integrating small fragments of foreign DNA into specialized genomic loci called clustered regularly interspaced short palindromic repeats (CRISPRs). Subsequent infections trigger an adaptive immune response, which relies on both CRISPR RNAs (crRNAs) and CRISPR-associated (Cas) proteins to identify and destroy invading DNA. In E. coli, the crRNA/Cas ribonucleoprotein surveillance complex is referred to as Cascade. Cascade recognizes foreign DNA targets via crRNA-DNA base pairing and subsequently recruits a trans-acting nuclease/helicase, Cas3, for degradation of the targeted DNA. To fully dissect the mechanistic features of target recognition and degradation by Cascade/Cas3, we have used a combination of single molecule and bulk methods. We reveal crucial features in DNA that Cascade senses to locate and recognize complementary target sequences within the larger context of genomic DNA. Further, we uncover the key elements necessary for Cascade-dependent recruitment of Cas3. Finally, by directly visualizing Cas3 as it unwinds and cleaves viral DNA, we characterize the degradation machinery in exceptional detail, providing fundamental insights into the mechanism of CRISPR-based immunity. Importantly, by comparing these results with our recent findings for another CRISPR-Cas system, Cas9 in S. pyogenes, we can begin to understand the evolution of DNA targeting machineries in related RNA-based adaptive immune systems.

2175-Plat

Selective Acetylation Reveals Distinct Roles of Histones H3 and H4 in Nucleosome Dynamics - a FRET Study

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Histone tails and their posttranslational modifications play a crucial role in controlling genetic activity through alterations of nucleosome structure. Whether or not histone tails regulate DNA accessibility independently of each other or in a concerted fashion is currently under debate. Here we studied the structure-defining properties of selective histone acetylation and point mutations in the H4 tail in a combined bulk FRET - single molecule FRET assay. Nucleosome unwrapping was monitored by FRET between the linker ends of the DNA, while FRET experiments at an internal DNA site in the H2A/H2B binding region reported on nucleosome disassembly.

By analysis of nucleosome unwrapping, structural heterogeneity during salt-induced disassembly and dimer exchange between nucleosomes we show that histones H3 and H4 assume significantly different roles in controlling nucleosome architecture. H4-acetylation opposes destabilization by H3-acetylation and reduces linker DNA unwrapping and dimer exchange at higher ionic strength, whereas its influence on nucleosome structure at physiological salt is minute. We found no increase in unwrapping when H3 and H4 were acetylated simultaneously, which challenges the idea of cooperativeness between tails that was observed for truncated H3 and H4. Our data suggest that the effect of lysine acetylation is not cumulative in nature but shows strong histone specificity. The specific role of the H4 tail was finally probed by comparing the effect of point mutations or acetylation of selective lysine residues at positions 5,8,12 and 16.

Regardless of the state of acetylation nucleosomes disassemble via an intermediate state, which is suppressed at higher nucleosome concentration, confirming our proposed model of step-wise disassembly.

Platform: Calcium Fluxes, Sparks, and Waves

2176-Plat

Decomposition of a Calcium Spark in Cardiac Myocytes

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Ca^{2+} sparks are the elementary SR Ca^{2+} release events in heart muscle. Spontaneous and triggered Ca^{2+} sparks are usually centered on a junctional sarcoplasmic reticulum (jSR) across a 15 nm "subspace" gap from a transverse tubule (TT) at the Z-disk. The jSR-TT junction contains ryanodine receptor (RyR2) clusters of a variable number of RyR2s and diverse organizations. To determine if Ca^{2+} sparks may reveal a substructure that reflects the jSR-RyR2 organizational variability, we have examined rabbit ventricular myocytes and imaged $[Ca^{2+}]$ in living myocytes using a confocal microscope. The cytosol was loaded with the $[Ca^{2+}]_i$ indicator rhod-2 and the SR loaded with the low affinity $[Ca^{2+}]_{SR}$ indicator fluo-5N. This method has allowed the detection of Ca^{2+} blinks (the local SR Ca^{2+} depletion during a Ca^{2+} spark) and Ca^{2+} sparks simultaneously. Furthermore, this method makes it possible to image the recently described set of small, sub-spark events, the quarky SR Ca^{2+} release or QCR. The spatial profiles of Ca^{2+} sparks and Ca^{2+} blinks has thus revealed a sub-structure within a single Ca^{2+} spark. The images suggest that Ca^{2+} sparks could be composed of a central Ca^{2+} release site with surrounding small release site elements (RS-elements). These RS-elements may be asymmetrically distributed around the central site. That high concentrations of EGTA (30 μM) prevented the activation of the RS-elements suggests that these RS-elements or QCRs are activated by Ca^{2+}-induced Ca^{2+}-release (CICR) triggered from the central site. How the underlying structures may change in diverse diseases may be important in broadening our understanding of both arrhythmogenesis and heart failure, diseases widely associated with altered Ca^{2+} signaling, $[Ca^{2+}]_i$ stability and altered cellular ultrastructure.

2177-Plat

Examination of Single Channel RyR Behavior from Long-Lasting Ca^{2+} Sparks

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Calcium sparks are crucial to cardiac excitation-contraction coupling and are due to calcium-induced calcium release via ryanodine receptors (RyRs) located on the sarcoplasmic reticulum (SR). A calcium spark results from the concerted opening of a cluster of RyRs, which generally closes by a robust mechanism as shown by stereotypic spark duration (~40 ms). However, much longer calcium sparks (up to seconds) can be seen in cells isolated from an animal models of heart failure (1) and when RyRs have been pharmacologically inhibited (2). The mechanism underlying such long-lasting events is unclear. It has been suggested that a lack of local SR calcium depletion (from a combination of decreased release flux and increased SR calcium buffering and/or refilling) is important (2), however it has also been suggested that the activity of RyR sub-clusters (located 1 ms =~1 um apart) may also sustain release (1). We examined calcium sparks in cells treated with tetracaine to partially inhibit RyRs. Spark-like and long-lasting events occurred at the same location in a line-scan image, suggesting a single release site is able to exhibit both types of behavior. Analysis of long-lasting release events (mean duration = 315 ms) yielded results consistent with a mechanism that involves a reduction in RyR availability and there was no detectable change in the location of the origin of release. These conclusions are supported by a computer model that was able to reproduce long-lasting events when the number of stochastically gating RyRs available was reduced and SR calcium was increased. Large alterations in RyR cluster geometry were not required, but the geometric organization of RyRs in the cluster plays an important role.

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